

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
2 May 2002 (02.05.2002)

PCT

(10) International Publication Number
WO 02/34777 A1

(51) International Patent Classification⁷: C07K 14/47, (74) Agents: MINOJA, Fabrizio et al.; Bianchetti Bracco Minoja S.r.l., Via Rossini, 8, I-20122 Milano (IT).
A61K 39/385, A61P 25/28

(21) International Application Number: PCT/EP01/12242

(22) International Filing Date: 23 October 2001 (23.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI2000A002299 24 October 2000 (24.10.2000) IT

(71) Applicant (for all designated States except US): CHIESI FARMACEUTICI S.P.A. [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): GHIRARDI SILVIA [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT). ARMANI, Elisabetta [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT). AMARI, Gabriele [IT/IT]; Via Palermo, 26/A, I-43100 Parma (IT). PUCCINI, Paola [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT). IMBIMBO, Bruno [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT). VILLETTI, Gino [IT/IT]; Via Palermo 26/A, I-43100 Parma (IT).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/34777 A1

(54) Title: FUSION PROTEINS AS IMMUNIZATION TREATMENTS OF ALZHEIMER'S DISEASE

(57) Abstract: The present invention is related to fusion proteins (A β -Hsp) (III) and their use in the treatment or prophylaxis of disorders associated with an accumulation of β -amyloid, specifically in patients with Alzheimer's disease. Said fusion proteins (III) are derived from the condensation of β -amyloid protein or fragments thereof (A β) (I) with a heat shock protein (Hsp) (II) according to the following pathway: (Formula).

FUSION PROTEINS AS IMMUNIZATION TREATMENTS OF
ALZHEIMER'S DISEASE

The present invention is related to fusion proteins derived from the condensation of β -amyloid protein or a fragment thereof and a heat shock protein, to the pharmaceutical preparations that contain them and to their use in the treatment or prophylaxis of disorders associated with the 5 overproduction of β -amyloid, specifically, in patients with Alzheimer's disease.

BACKGROUND

Alzheimer's disease is a growing problem in developed countries. This disorder accounts for approximately 170,000 deaths in the United States every 10 year, placing it in third place (7.1% of total deaths) as cause of death (Ewbank DC, *Am J Public Health* 1999; 89: 90-92). The yearly cost of Alzheimer's disease in the United States is estimated to be 100 billion dollars (Schumock GT, *Am J Health Syst Pharm* 1998; 55, Suppl. 2, : S17-S21). The cost of treating each patient is estimated to be 195,000 dollars, with a significant 15 amount of this cost being attributed to the loss of productivity of the patient and caregiver and to costs sustained by the family. The average annual cost for the care of patients with a mild, moderate or severe form of the disorder, is approximately 19,000, 31,000 and 37,000 dollars, respectively. The availability of drugs capable of slowing the development of the disease would 20 lead to an estimated yearly savings of approximately 25,000 dollars per patient in medical assistance costs alone (Leon J *et al*, *Health Aff* 1998; 17: 206-216).

From an anatomic point of view, Alzheimer's disease is characterized by an atrophy of the cerebral cortex and by a massive loss of cortical neurons 25 and cholinergic projections made by the nucleus basalis towards the cortex.

From a histopathologic point of view there is a diffuse presence of extracellular and perivascular neuritic plaques and intracellular neurofibrillary tangles in the cerebral parenchyma of Alzheimer patients.

In addition to these histopathologic lesions, the primary 5 neurotransmitter acetylcholine as well as other neurotransmitters such as serotonin, noradrenaline, dopamine, glutamate and substance P are degraded (Price DL *et al*, *Ann NY Acad Sci* 1985; 457: 35-51). Acetylcholinesterase inhibitors have been employed in an attempt to increase acetylcholine levels in the brain, but this pharmacological approach to the problem has yielded 10 modest clinical results and has not had a significant impact on the natural history of the disorder (Davies P, *JAMA* 1999; 281: 1433-1434). For this reason in recent years research has focused its efforts on understanding the mechanism behind the formation of the characteristic lesions found in the brain of Alzheimer patients: neuritic plaques and neurofibrillary tangles.

15 Neuritic plaques are composed mainly of aggregates of a protein with 39-43 amino acid residues known as β -amyloid (Selkoe DJ, *J Neurochem* 1986; 46: 1820-1834). The β -amyloid protein is a derivative of a complex transmembrane glycoprotein (Kang J *et al*, *Nature* 1987; 325: 733-736) known as amyloid precursor protein (APP), and comprises a small part of the 20 extracellular domain. The initial step of the metabolic pathway of APP involves the α -secretase enzyme that cleaves out APP within the β -amyloid sequence (Esch FS *et al*, *Science* 1990; 248: 1122-1124) allowing for the release of its transmembrane fragment. This metabolic pathway is not amyloidogenic because it precludes the formation of β -amyloid and leads to 25 the release of APP α that appears to exert neuroprotective activity (Mattson MP *et al*, *Neuron* 1993; 10: 243-254). The metabolic pathway, defined amyloidogenic, that leads to the formation of β -amyloid is due to the enzyme β -secretase that releases APP β plus a 12 kDa protein fragment, which in turn

is cleaved by the γ -secretase enzyme (Esler WP *et al*, *Science* 2001; 293:1449-1454) giving way to soluble A β .

Mutations of APP and of other recently identified proteins such as presenilins (PS-1 and PS-2), associated with familial forms of early onset 5 Alzheimer, result in an overproduction of β -amyloid, or at least of its 42 amino acid form (Sherrington R *et al*, *Nature* 1995; 375: 754-760, Rogaev EI *et al*, *Nature* 1995; 376: 775-778). In addition, several studies have shown that the overproduction of β -amyloid may be promoted in the presence of apolipoprotein E type 4 allele, a known risk factor implicated in Alzheimer's 10 disease. These data seem to indicate that genetic changes commonly associated with Alzheimer's disease are involved in the increased production of β -amyloid protein.

There are several variants of the β -amyloid protein, derived by proteolytic cleavage from APP, that differ only in terms of the C-terminal 15 domain: A β ₃₉, A β ₄₀, A β ₄₂ and A β ₄₃. The 40 and 42 aminoacid forms are found predominantly in the extracellular neuritic plaques, while the 39 residue peptide is the predominant component found in cerebrovascular deposits (Mori H *et al*, *J Biol Chem* 1992; 267: 17082-17086, Prelli F *et al*, *Biochem Biophys Res Commun* 1988; 151: 1150-1155). The amino acid sequence 20 (primary structure), characterized by the presence of some hydrophobic amino acids, is such that the amyloid protein may take on both an α -helical and β -sheet conformation. Even though the two structures are approximately isoenergetic in the diagram of Ramanchandran, the protein adopts the β -sheet conformation in Alzheimer's disease for reasons not yet clear. Contrary to the 25 α -helix form that is soluble in the cerebral parenchyma, the β -sheet form gives origin to the neurotoxic aggregates of the protein. β -amyloid fibrils are believed to be formed by two β -sheet filaments, derived from protein folding, and linked by an α -helix turn (Soto C *et al*, *J Neurochem* 1994; 63: 1191-

1198). The deposition of β -amyloid aggregates is thus highly correlated with its secondary β -sheet structure (Soto C *et al*, *Neurosci Lett* 1995; 200: 105-108). The fibrillogenesis of the β -amyloid protein involves a two-stage polymerization process: an initial "nucleation" process followed by "polymer elongation" that gives origin to the protofibrils (Jarrett JC *et al*, *Cell* 1993; 73: 1055-1058). The alignment of the protofibrils generates the β -amyloid fibrils.

5 The correlation among histopathologic lesions, brain cell death and cognitive deficiency in Alzheimer's disease is the basis of different etiology theories for the pathogenesis of Alzheimer's disease. The amyloid theory sustains that β -amyloid deposits in the brain of Alzheimer patients cause the disorder. Important evidence sustains this theory: (i) autopsies performed on the brain of Alzheimer patients consistently reveal β -amyloid deposits (McKhann G *et al*, *Neurology* 1984; 34: 939-944); (ii) all of the dominating autosomic mutations associated with the familial early onset forms of 10 Alzheimer's disease are characterized by an overproduction of β -amyloid 1-42 (Citron M *et al*, *Nature* 1992; 360: 672-674, Scheuner D *et al*, *Nat Med* 1996; 2: 864-870, Suzuki N *et al*, *Science* 1994; 264: 1336-1340, Citron M *et al*, *Nat Med* 1997; 3: 67-72, Borchelt DR *et al*, *Neuron* 1996; 17: 1005-1013); (iii) the formation of β -amyloid plaques precedes the symptoms of the disease 15 (Lippa CF *et al*, *Lancet* 1998; 352: 1117-1118). (4) The clearance of β -amyloid1-42 in the brain of patients appears to be reduced (Motter R *et al*, *Ann Neurol* 1995; 38: 643-648).

20 An important step in the research of Alzheimer's disease was the development and use of transgenic mice carrying one or more familial mutations of APP (Hsiao K *et al*, *Science* 1996; 274: 99-102, Games D *et al*, *Nature* 1995; 373: 523-527, Lamb BT *et al*, *Hum Mol Genet* 1997; 6: 1535-1541, Mucke L *et al*, *Ann N Y Acad Sci* 1996; 777: 82-88). The overproduction of APP, the extent of β -amyloid deposits in the brain, neuritic

dystrophy, and synapse loss observed in this animal model was of the same order as that observed in patients with Alzheimer's disease. There has been a recent report of a transgenic mouse with nerve growth factor (NGF) antibodies showing cortical and hippocampal accumulations of both β -amyloid protein 5 and τ hyperphosphorylate protein (Ruberti F *et al*, *J Neurosci* 2000; 20: 2589-2601). The aged anti-NGF transgenic animals showed an extensive neuronal loss in the cortex, cholinergic deficiency in the nucleus basalis and cognitive and behavioral deficiency (Capsoni G *et al*, *Proc Natl Acad Sci USA* 2000; 97: 6826-6831). These animals are extremely useful in testing new therapeutic 10 theories that could modify the natural history of the disorder.

Currently several therapeutic approaches are being developed based on the amyloid theory of Alzheimer's disease. One of these is related to identifying selective γ -secretase (Wolfe MS *et al*, *J Med Chem* 1998; 41: 6-9, Higaki J *et al*, *Neuron* 1995; 14: 651-659) or β -secretase (Sinha S *et al*, *Proc 15 Natl Acad Sci USA* 1999; 96: 11049-11053) inhibitors that could block the formation of β -amyloid protein and reduce amyloid plaques, thus decreasing brain cell dysfunction and death. Another approach involves the inhibition of β -amyloid aggregates with products that modify the secondary structure of the protein or that bind to specific regions of the protein itself (Soto C *et al*, *Nat 20 Med* 1998; 4: 822-826, Poduslo JF *et al*, *J Neurobiol* 1999; 39: 371-382, *Biochemistry* 1999; 38: 6791-6800).

Recently, the Athena Neuroscience international patent application WO 99/27944, claimed the same β -amyloid protein or its active fragment, as an agent able to induce an immunogenic response to β -amyloid, in the 25 prophylaxis and treatment of amyloidogenic disorders and in Alzheimer's disease in particular. Schenk *et al.* have demonstrated that immunization using β -amyloid1-42 in transgenic mice harboring a mutant version of APP (PDAPP) inhibits the formation of amyloid plaques and histopathologic

lesions (Schenk D *et al*, *Nature* 1999; 400: 173-177). The immunization of young animals results in the almost total absence of β -amyloid deposits in old age with a significant inhibition of neuritic dystrophy (a marker of neuronal dysfunction) and of astrogliosis (a marker of cerebral inflammation). The 5 immunization of aged animals (11-12 months) that had already developed β -amyloid plaques prevented additional histopathologic lesions and even led to their partial regression. The residual amyloid plaques are actively metabolized by microglia cells, suggesting that the immunization may, in addition to preventing the deposit of new amyloid, lead to the elimination of 10 already existing deposits. The clearance effect of the β -amyloid protein is thought to take place through a mechanism of phagocytosis exerted by microglia monocytes. Other reports confirmed these initial findings (Janus C *et al*, *Nature* 2000; 408: 979-982, Morgan D *et al*, *Nature* 2000; 408: 982-985) thus supporting the idea of developing a protein β -amyloid1-42 vaccine for 15 Alzheimer patients.

A further understanding is needed before some of these aspects may be applied to Alzheimer's disease (St George-Hyslop PH *et al*, *Nature* 1999; 400: 116-117). First of all it has been observed that in order to achieve the same effect as that observed in mice models, high concentrations of β -amyloid1-42 20 antibodies are necessary. Thus, the immune response induced by a vaccine may not be sufficient in man. In addition, contrary to mice models, the phenomenon of tolerance may occur in man, whereby the human immune system may be unable to react against a protein belonging to its own organism (β -amyloid 1-42). Further, it would be preferable to administer the antigen as 25 a vaccine in the dissociated form and in such a way as that is able to maintain its secondary soluble structure: endogenous factors could indeed favor transconformation and/or denaturization processes that have a negative impact on the duration of the activity. Although in WO 99/27944, a generic

awareness is reported about the conformational changes in the immunogen that could affect the qualitative form of the response, in the examples (p.25, lines 27-30), the immune response in animal was tested by using the aggregated form of A β ₄₂, i.e. a mixture of oligomers in which the monomeric 5 units are held together by noncovalent bonds (p.12, lines 32-34). Upon injection, said aggregated form might give rise to larger agglomerates which could locally precipitate so being not able of eliciting their immunogenic activity. The deposit of such agglomerated could also give rise to granuloma and fastidious inflammations at the administration site.

10 Finally, it would be more advantageous to provide compositions containing the antigen able to induce a sufficient immune response in man without the use of an adjuvant and in particular without the use of the Freund's adjuvant. Although WO 99/27944 it is also generally directed to compositions free of Complete Freund's adjuvant and notwithstanding that, in 15 the specification examples with alternative adjuvants are reported, (par. IX p. 66) the best results have been achieved with an immunization treatment envisioning first the use of emulsions containing A β ₄₂ peptide (in the aggregated form) and the Complete Freund's adjuvant followed by the use of emulsions containing Incomplete Freund's Adjuvant (see Fig. 14). Antibody 20 response against A β ₄₂ peptide alone was negligible (see again Fig. 14). On the other hand, the complete Freund's adjuvant unfortunately exhibits a series of toxic effects, some of which are serious (Broderson JR, *Lab Anim Sci* 1989; 39: 400-405, Claassen E *et al*, *Res Immunol* 1992; 143: 478-483, Mancardi S *et al*, *Exp Cell Res* 1999; 246: 368-375, Andersen KE *et al*, *Contact 25 Dermatitis* 1996; 35: 127-128, Haak T *et al*, *Clin Exp Rheumatol* 1996; 14: 633-641, Rabchevsky AG *et al*, *Brain Res* 1999; 832: 84-96). Some concerns exists about the use of the incomplete form as well; moreover, although it is better tolerated in man, it seems unable to increase the cellular immune

response (Jensen FC *et al*, *Adv Drug Deliv Rev* 1998; 32: 173-186).

In view of the problems outlined above, it would be highly advantageous to provide compositions free of adjuvant to be used in the treatment or prophylaxis of disorders associated with an accumulation of 5 β -amyloid, such as Alzheimer's disease, containing as vaccine a form of dissociated A β protein or fragment thereof either able either to maintain the soluble secondary structure and to elicit a sufficient immune response in man.

Heat shock proteins (Hsp), also known as stress proteins, were described for the first time in *Drosophila* as proteins that are released 10 following heat shock. Later it was found that their release is also related to stress or traumas of other types (hypoxemia, chemical, etc.). Hsp are classified according to at least four categories based on size: Hsp90 (90 kDa), Hsp70 (70 kDa), Hsp60 (60 kDa) and small Hsp (< 20 kDa). Hsp act as an intracellular chaperone to proteins, facilitating the transition among different 15 compartments, modifying the secondary and tertiary structure and assembly of multimers (Beckmann RP *et al*, *Science* 1990; 248: 850-854). Recently, an important humoral and cellular immunogenic role has also been attributed to Hsp (Mizzen L, *Biotherapy* 1998; 10: 173-189). Their strong immunostimulant properties have made them potentially significant 20 therapeutic anti-infection and cancer agents. Hsp 90 and Hsp70 have recently been assessed as anti-cancer vaccines.

It has been demonstrated that the neurotoxicity of β -amyloid and τ hyperphosphorylate proteins induces an increase in Hsp, especially Hsp 70 in the brain of Alzheimer patients (Perez N *et al*, *Mol Brain Res* 1991; 11: 249-254, Johnson G *et al*, *Ann N Y Acad Sci* 1993; 695:194-197, Yoo BC *et al*, *J Neural Transm Suppl* 1999; 57: 315-322). It has also been shown that the expression levels of Hsp 27 increase in degenerative astrocytes in brain areas rich in senile plaques during the inflammatory process (Renkawek K *et al*,

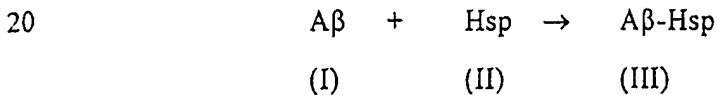
Acta Neuropathol 1994; 87: 511-519).

In an attempt to increase the immune response of the antigen, fusion proteins (Ag-Hsp) deriving from a covalent bonding of an antigen protein (Ag) and a heat shock protein (Hsp) have recently been synthesized (Suzue K 5 *et al, Proc Natl Acad USA* 1997; 94: 13146-13151). Contrary to protein antigens that activate the T helper CD4+ lymphocytes and generate a humoral immune response, these Ag-Hsp fusion proteins activate the T cytotoxic CD8+ lymphocytes, thus generating a cellular immune response (Huang O *et al, J Exp Med* 2000; 191: 403-408).

10 The role of Hsp in brain of patients with Alzheimer's disease has also been recently investigated (Yoo BC *et al J Neural Trasm* 1999; 57, 315-322).

OBJECT OF THE INVENTION

The compounds of the present invention are fusion proteins (A β -Hsp) (III) derived from the covalent bonding of protein β -amyloid or fragment 15 thereof (A β) (I) and a heat shock protein (Hsp) (II). Fusion proteins A β -Hsp (III) exert immunization activity against the β -amyloid protein and are thus useful in the treatment of all pathologic conditions that are characterized by an overproduction of β -amyloid and by β -amyloid deposits, in particular in Alzheimer's disease.



According to recent reports in literature (Schenk D *et al, Nature* 1999; 400: 173-177), the immunization achieved following the systemic administration of human (A β ₄₂) β -amyloid protein (I) together with a 25 suitable immune coadjuvant (Freund) to transgenic mice with a spontaneous accumulation of β -amyloid in the brain, prevents the deposition of β -amyloid in young animals and blocks additional deposition of the protein in aged animals who have already developed senile plaques. In addition to this

inhibitory effect exerted against β -amyloid deposition in the brain, histopathologic abnormalities (neuritic and astrogliosis dystrophy) are also attenuated.

The products (III), and the pharmaceutical preparations containing them, induce an immune response to the β -amyloid protein and inhibit and prevent neuropathologic symptoms induced by the aggregates of the protein, and as such, are useful in the treatment and in the prophylaxis of Alzheimer's disease. The specific heat shock protein that is bonded to the peptidic fraction of the β -amyloid protein may be appropriately modulated in order to stabilize the secondary and tertiary structure of the peptidic fragment of the β -amyloid protein, to increase the immunogenic potential and to favor cellular transition. As demonstrated in the following example, the fusion protein obtained by coupling $A\beta_{42}$ with Hsp70 is able to induce an immune response comparable to that of $A\beta_{42}$ in the presence of the Freund's adjuvant, suggesting that compositions containing said agents as vaccine could be used for inducing a therapeutic immune response in patients affected by Alzheimer's disease, without the need of an adjuvant.

There is no suggestion in the prior art for using heat shock proteins for enhancing the immune response of $A\beta$ protein or fragments thereof in a so significant way to envision the possibility of using compositions free of adjuvants.

EP 526,511 proposes administration of homeopathic dosages (less than or equal to 10^{-2} mg/day) of $A\beta$ to patients with pre-established Alzheimer's disease. In WO 99/27944, it is claimed, among others, that $A\beta$ peptides can be linked to suitable carriers such as serum albumin, choleric toxin, immunoglobulin or attenuated diphtherotoxin CRM 197 (p. 20, lines 1-7) which may favor release and/or immune response; it is also generically stated that immunogenic peptides can also be expressed as fusion proteins with

carriers (p. 20, lines 33-36), but there no mention of heat shock proteins and, overall, there is no suggestion that the latter ones could be used for stabilizing the secondary structure of A β and augmenting its immune response to the level which can be obtained in the presence of the Complete Freund's adjuvant.

5 Monomeric A β peptides expressed as fusion proteins are also the subject-matter of EP 641861 but even in this there is no mention of Hsp.

WO 94/29459 refers to the use of stress proteins for modulating the immune response. Among others, the invention is also directed to fusion protein comprising a stress protein fused to a protein against which an immune 10 response is desired but fusion proteins between A β and Hsp are nor mentioned, neither the less exemplified.

15 The β A-fusion protein (III) can include any of the naturally occurring β -amyloid proteins or a peptidic fragments thereof, and any Hsp chosen among the heat shock proteins with a low (Hsp25, Hsp27, Hsp28, etc.) or those with a high molecular weight (Hsp60, Hp70, Hsp90, etc.).

Particularly preferred are the human forms of A β protein, i.e. A β ₃₉, A β ₄₀, A β ₄₁, A β ₄₂ and A β ₄₃. Both the A β peptidic fragment and the heat shock protein can be appropriately chosen to obtain: i) the highest immunogenic response possible; improved tolerability; the most stable structure.

20 This invention also includes all the possible isomers of the compounds having the general formula (III) and their mixtures.

The present invention also includes the salts of those compounds having the general formula (III) that possess groups that can be salified, in particular the carboxylic and amino groups.

25 The physiologically tolerated salts are for example, in the case of molecules containing acid groups, salts of alkaline metals or alkaline-earth, such as sodium, potassium, lithium, calcium, magnesium, or salts formed with organic or amino acid amines, such as arginine. In the case of molecules

containing basic groups the salts can comprise inorganic acids, such as hydrochloric acid and sulfuric acid, and mono and dicarboxylic organic salts, such as acetic acid, tartaric acid and methansulfonic acid.

Although they do not belong to the preferred embodiment, the present 5 invention also relates to preparations containing the fusion protein of general formula (III) and immune adjuvants such as that of Freund (complete or incomplete), Jung, Gerbu, QS-21.

A further embodiment of the invention concerns the nucleic acid sequence encoding the fusion protein .

10 The fusion proteins according to the invention can be prepared with the aid of appropriate recombinant vectors in host cells. The techniques of constructing vectors, transforming cells, bringing about expression of the fusion proteins in the transformed cells and isolating and purifying the expressed proteins are known *per se* to the person skilled in the art. 15 Advantageously, the A_β peptide can be linked to the heat shock protein by suitable linker amino acids.

Expression can take place not only in bacteria but also in a large number of host systems such as, for example, in mammalian, yeast and insect cells. The DNA constructs suitable for the various host cells are synthesized 20 by known methods and incorporated in the genome of the host cells with appropriate control sequences in a conventional way.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological 25 state of the patient, other medicament administered and whether treatment is prophylactic or therapeutic. The amount of fusion protein for administration can vary from 10 µg to 20 mg per injection, preferably from 100 µg to 10 mg.

A typical regimen consists of an immunization (priming induction)

followed by booster injections at suitable intervals.

The fusion proteins of the invention can be administered by any route for prophylactic and/or therapeutic treatment. The most typical route of administration are subcutaneous and intramuscular injections.

5. The pharmaceutical compositions containing the fusion proteins of the invention can also include a variety of other pharmaceutically acceptable components depending on the intended mode of administration.

The following examples illustrate in detail the invention.

Example 1

10 **PREPARATION OF THE TEST MATERIALS, NAMELY THE FUSION PROTEIN A_β₄₂-HSP70 AND THE A_β₄₂ PEPTIDE**

Materials and Methods

Expression Vector Constructs

The DNA fragment containing the *Mycobacterium tuberculosis* Hsp70 sequence was synthesized by PCR technique using the genomic DNA extracted from the *M. tuberculosis* HR37Rv strain as template. The upstream primer overlapping the AUG start codon (Hsp70-up) contains the *BamHI* and *NcoI* sites, while the downstream primer overlapping the UGA stop codon (Hsp70-dw) contains the *XhoI* site. The amplified DNA was then digested 15 with the *BamHI* and *XhoI* enzymes and cloned in a pGEM11 Z(f+) (Promega). A positive clone (p11[Hsp70]) was isolated and fully sequenced. The DNA fragment encoding for the *Homo sapiens* amyloid beta peptide 1-42 (A_β₄₂) was synthesised by the annealing of the oligos Beta-1 and Beta-2. The Beta1/2 double strand DNA fragment contains: the *HindIII* site in the 5' of the 20 untranslated sequence, the *NdeI* site that overlap the AUG start codon and the *BamHI* site coding for the linking aminoacids (Gly-Ser) between the A_β₄₂ and the Hsp70 sequence. The Beta1/2 DNA fragment was digested with *HindIII* 25 and *BamHI* enzymes, and then cloned in the p11[Hsp70] vector. A positive

clone (p11[A β ₄₂-Hsp70]) was isolated and fully sequenced. The p11[A β ₄₂-Hsp70] was digested with the enzymes NdeI and XhoI, and the 2 Kb band coding for the chimeric protein A β -Hsp70 was then cloned in the pET24a plasmid (Novagen). A positive clone (p24[A β ₄₂-Hsp70]) was isolated 5 and fully sequenced.

Protein Purification

The *Escherichia coli* BL21/DE3 strain was transformed with the p24[A β -Hsp70] vector expressing the chimera A β ₄₂-Hsp70 and with the pET24a as control. Overnight cultures of the transformants were diluted 1/100 10 in LB medium containing 30 μ g/ml of Kanamycin. The cultures were grown to an OD_{600nm} of 1 and recombinant protein production was induced by addition to the cultures of isopropylthiogalactoside (IPTG) at the final concentration of 1 mM, for a total of four hours. The A β ₄₂-Hsp70 protein formed inclusion bodies and was then purified as follows. The cells were harvested by 15 centrifugation at 10,000 rpm in a JA10 Beckman rotor. The cell pellet was resuspended in lysis buffer A and incubated at 30°C for 15'. Then an equal volume of lysis buffer B was added and incubated at 37°C for 15'. The cells were sonicated and then pelleted at 12,000 rpm in a JA17 Beckman rotor at 4°C for 15'. The pellet were resuspended and pelleted three times with the 20 buffer C, D and E, respectively. The final pellet was resuspended in buffer F and then centrifuged as previously described the supernatant were frozen at -20°C. Proteins were refolded by dilution 1:100 in the refolding buffer for 16 hours at 4°C in agitation. The refolded solution was filtered with Millipore 0,45 μ m filters and then it was concentrated of 20 fold by ultra-filtration with 25 a Pellicon XL Cellulose 10K (Millipore) using a Labscale apparatus (operative pressures P_{in} = 20-30 Psi; P_{out} = 12-15 Psi) (Millipore).

The ultra-filtered solution was buffer exchanged using a G25 HiPrep 26/10 column (Amersham Pharmacia Biotech) using the buffer G, the protein

fraction was used for the ATP affinity chromatography. A column of ATP-agarose (Sigma) was equilibrated in buffer G plus 0.2% TritonX100 and 100 mM NaCl. Protein was loaded on the ATP column and subsequently the column was rinsed with: buffer G plus 0.2% Triton X100 and 600 mM NaCl; 5 buffer G plus 100 mM NaCl; buffer G plus 100 mM NaCl and 5 mM ATP. The protein A β ₄₂-Hsp70 was eluted with buffer G plus 100 mM NaCl and 25 mM ATP. A β ₄₂-Hsp70 protein was polished by gel filtration using a HiLoad 16/60 Superdex 200 prep grade column (Amersham Pharmacia Biotech), the fractions containing the A β ₄₂-Hsp70 protein were pooled and dialyzed against 10 PBS and then stored at -80°C.

Lysis buffer A: 50 mM Tris-HCl, pH = 8; 25 % sucrose; 1 mM EDTA, pH = 8; 0,1% NaN₃; 10 mM DTT; 5 mM MgCl₂; 0.5 mg/ml Lysozyme (SIGMA); 0.4u/ml DNAsi (EPICENTRE).

Lysis buffer B: 50 mM Tris-HCl, pH = 8; 1 % Triton X100; 100 mM NaCl; 15 0,1% NaN₃; 10 mM DTT; 1 mM PMSF.

Buffer C: 50 mM Tris-HCl, pH = 8; 0.5 % Triton X100; 100 mM NaCl; 1 mM EDTA, 0,1% NaN₃; 1 mM DTT.

Buffer D: 50 mM Tris-HCl, 1 M NaCl.

Buffer E: 50 mM Tris-HCl, 1 M urea.

20 Buffer F: 50 mM Tris-HCl pH=8, 7M urea.

Buffer G: 100 mM Tris-HCl pH = 7.5; 4 mM MgCl₂.

Refolding buffer: 500 mM L-Arginina:105.335 g/litro; 100 mM Tris-HCl pH = 8; 2 mM EDTA; 10mM L-Cysteine; 0.5 mM L-Cystine.

Results

25 The production of the chimeric protein A β ₄₂-Hsp70 obtained from the fusion between the human amyloid beta peptide 1-42 (A β ₄₂) and the *Mycobacterium tuberculosis* Hsp70 protein was achieved.

The synthetic nucleotide sequence of the A β ₄₂ peptide was fused with

Hsp70 gene of the *M. tuberculosis* (Figure 1) and then cloned into the pET24a vector. This vector allows the expression of high levels of recombinant protein using the T7 expression system (Studier FW *et al.*, *Methods Enzymol* 1990, 185: 60-89). The $\Delta\beta_{42}$ -Hsp70 fusion protein was found to be expressed at very high levels in *E. coli* (Figure 2). The $\Delta\beta_{42}$ -Hsp70 protein was purified as inclusion bodies and subsequently refolded. The protein was further purified by ATP affinity chromatography and gel filtration using a Superdex 200 prep grade column. The purity of the recombinant protein was assessed by SDS-PAGE (Figure 2): lanes 1, 2, 3 are cell lysates of the BL21/DE3 cells 5 transformed with the empty vector, p24[$\Delta\beta_{42}$ Hsp70] prior to IPTG induction and after 4 hours of induction, respectively. Lane 4 represents the refolding of the $\Delta\beta_{42}$ Hsp70 protein, and lane 5 the purified $\Delta\beta_{42}$ Hsp70 protein (indicated 10 by the arrow). (Visualisation by Coomassie staining).

Oligonucleotides Table

15

Hsp70-up	GGG ATC CAT GGC TCG <u>GGG</u> GGT C
HSP70-dw	CCG CTC GAG TCA CTC <u>GGG</u> C C CCG
BETA-1	CCC AAG CTT CCA TAT GGA TGC AGA ATT CCG ACA TGA CTC AGG ATA TGA AGT TCA TCA TCA AAA ATT GGT GTT CTT TGC AGA AGA TGT GGG T T C AAA CAA AGG TGC AAT CAT TGG ACT CAI GGT GGG CGG TGT TGT CAT AGC GGG ATC CAT GGC TC
Beta-2	GAG CCA TGG ATC CCG CTAT GCA CAA CAC CGC CCA CCA TGA GTC CAA TGA TTG CAC CTT TGT TGG AAC CCA CAT CTT CTG CAA AGA ACA CCA ATT TGT GAT GAT GAA CTT CAT ATC CTG AGT CAI GTC GGG ATT CTG CAT CCA TAT GGA AGC TGG GGG

Example 2**EVALUATION OF SYSTEMIC IMMUNE RESPONSES TO THE
FUSION PROTEIN A β ₄₂-HSP70****Material and Methods****5 Test Materials**

β -amyloid1-42 peptide (A β ₄₂) was bought from Unipeptides Inc. (Riverside, CA). The A β ₄₂-Hsp70 fusion protein was prepared according to the Example 1.

Animal Care

10 All animal experimentation were conducted in accordance to the current European guide lines for the use of animals in *in vivo* experimentation. The animals were housed under controlled conditions in group of ten per cage. On arrival to laboratory, each animal was assigned an identifying code. Periodic examinations of health were performed using microbiological, parasitological 15 and serological techniques aimed at identifying undesirable and pathogenic organisms.

Preparation of Test Materials

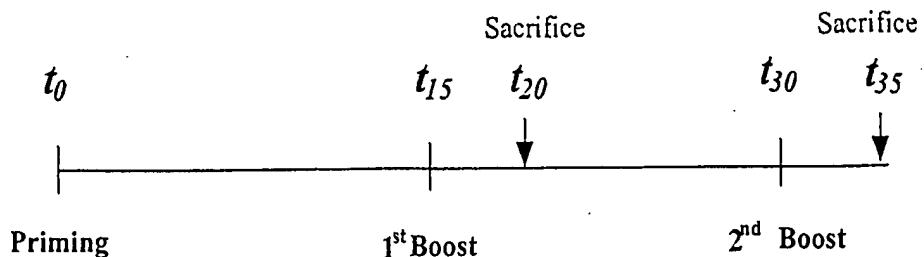
The test materials were used as lyophilised powders. Each test material was suspended at a concentration of 2 mg in 0.9 mL of sterile water, vortexed 20 and added with 100 μ L of 10 X phosphate buffered saline (PBS), where 1 X PBS is 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5. Suspensions were vortexed again and incubated overnight at 37°C for use the next day. Suspensions were prepared and codified by an independent operator in order to perform the study following a double blind procedure. Before 25 administration, the A β ₄₂ peptide was mixed with complete (*priming induction*) or incomplete (*subsequent challenges*) Freund's adjuvant in a ratio of 1:1 (v/v).

Animal Immunisation

The above processed test materials were administered subcutaneously at defined times (see below) to separate adult male mice (20-25 g, Balb/c, Charles-River, Lecco, Italy). Of noteworthy in this regard is that the choice of 5 the administration route and animal species, is based on the fact that: a) the subcutaneous route of administration is widely used in immunisation studies as the presence of specialised antigen-presenting cells (e.g. macrophages and dendritic cells) in the subcutaneous district induce a strong immunologic response; b) the mouse is a widely used animal species for studies on induced 10 immunological responses. In addition, all transgenic animal models of Alzheimer's disease currently available are in the mouse.

Mice were administered 10 or 100 µg of test material, according to the experimental scheme reported below. Each group was formed by 3-5 animals. Animals received, subcutaneously and in a dose corresponding to the assigned 15 group, the A β ₄₂ peptide mixed with complete Freund's adjuvant in a ratio of 1:1 (v/v) or alternatively the A β ₄₂-Hsp70 fusion protein alone (*priming induction*). Fifteen and 30 days after priming, animals received the first and second boost, respectively. Each time they were injected subcutaneously with A β ₄₂ peptide solution mixed with incomplete Freund's adjuvant in ratio of 1:1 20 (v/v) or with the A β ₄₂-Hsp70 fusion protein solution (*subsequent challenges* following priming induction). Animals of each group were sacrificed, by cervical dislocation, 5 days after receiving boost, i.e. 20 and 35 days after priming with antigen. Blood was collected for assessment of humoral immune responses (see below).

25 Also of noteworthy is that the priming induction, subsequent challenges and specimens collection were performed by an operator who was not informed about the nature of the administered solutions.



Assays

Antibody formation was initially evaluated in the sera prepared from the collected blood samples by immuno blot analyses. In the case of positive 5 results, antibody titres in the sera of the different animals were assessed via ELISA methodology.

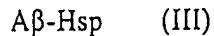
Results

The results in terms of anti- $\text{A}\beta$ serum IgG antibody titres at day 20 and day 35 in mice injected on day 1, day 15 and day 30 with 10 μg of $\text{A}\beta_{42}$ peptide (2 nmoles) or 100 μg of $\text{A}\beta_{42}$ -Hsp70 (1 nmole) fusion protein are plotted in Figure 3. Mice treated with $\text{A}\beta_{42}$ received complete Freund's adjuvant at day 1 and incomplete Freund's adjuvant at day 15 and day 30. Mice treated with the $\text{A}\beta_{42}$ -Hsp70 fusion protein did not receive Freund's adjuvant. The average titre (\pm standard error) is shown. Titres were defined as 15 the reciprocal of the dilution of serum giving one half the maximal optical density.

As it can be appreciated, the $\text{A}\beta_{42}$ -Hsp70 fusion protein, obtained by coupling $\text{A}\beta_{42}$ with Hsp70, is able to induce an immune response comparable to that of $\text{A}\beta_{42}$ in the presence of the Freund's adjuvant, suggesting that 20 compositions containing the fusion protein as vaccine could be used for inducing a therapeutic immune response in patients affected by Alzheimer's disease, without the need of an adjuvant.

CLAIMS

1. A fusion protein of the following formula:



5 its relative isomers, salt mixtures, in which $A\beta$ is a β -amyloid protein or a fragment thereof and Hsp is a protein belonging to the family of low or high molecular weight heat shock proteins.

2. A fusion protein according to claim 1 in which $A\beta$ is selected from β -amyloid1-39, β -amyloid1-40, β -amyloid1-42.

10 3. A fusion protein according to claim 1 or 2 in which Hsp is selected from Hsp 25, Hsp 27, Hsp 28, Hsp 60, Hsp 70, Hsp 90.

4. A nucleic acid sequence encoding for a fusion protein according to claims 1-3.

5. A pharmaceutical preparation having as its active ingredient a fusion 15 protein according to claims 1-3, in combination with diluents, and/or pharmacologically acceptable excipients and optionally an immune co-adjuvant.

20 6. A pharmaceutical preparation according to claim 5, in which the immune coadjuvant is selected from the Complete or Incomplete Freund's adjuvant, Jung, Gerbu and QS-21.

25 7. Use of a fusion protein- according to claims 1-3, in combination with diluents, pharmacologically acceptable excipients and optionally coadjuvants for the preparation of medicinal products in the treatment or prophylaxis of disorders associated with an abnormal production β -amyloid, in particular in Alzheimer's disease.

```

1 M D A E F R H D S G Y E V H H O K L V F F A E D V G S N K G
1 ATGGATGCAGAATTCCGACATGACTCAGGATAATGAAGTTCATCATCAAAAATTGGTGTCTTGCAGAAGATGTGGTTCAAAACAAAGT
31 A I I G L M V G G V V I A G S M A R A V G I D L G T T N S V
91 GCAATCATTGGACTCATGGTGGCGGTGTTGTCAAGCGGGATCCATGGCTCGTGGCTCGGATCCACCTCGGACCCACCAACTCGCTC
61 V S V L E G G D P V V V A N S E G S R T T P S I V A F A R N
181 GTCTCGGTTCTGGAAGGTGGCGACCCGGTCGTCGTCGCCAACCTCGAGGGCTCAGGACCCACCCCTCAATTGTGGCTTCGCCGCAAC
91 G E V L V G Q P A K N Q A V T N V D R T V R S V K R H M G S
271 GGTGAGGTGCTGTCGTCGCCAGCCGCAAGAACCCAGGCAGTACCGCAAGCTCGATGCCAGCGTCGCTCGGTCAAGGGACACATGGGAGC
131 D W S I E I D G K Y T A P T A R I L M K L K R D A E A
361 GACTGGTCCATAGAGATGACGGCAAGAAATACCCGGCCGGAGATCAGCGCCGCAATTCTGATGAAGCTGAAGGGCAGCGCCAGGCC
151 Y L G E D I T D A V I T T P A Y F N D A Q R Q A T K D A G Q
451 TACCTCGGTGAGGACATTACCGACGGCGTTACAGCACGCCGCCACTTCAATGACGCCAGCGTCAGGGCACCAGGACGCCGGCCAG
181 I A G L N V L R I V N E P T A A L Y G L D D K G E K E Q R
541 ATCGCCGGCCCTACGTCGTCGCCATCGTCACCGAGCCGACCCGGCCCTGGCTACGCCACAGGGAGAACGGGAGAACGGGAGACCGA
211 I L V F D L G G G T F D V S L L E I G E G V V E V R A T S G
631 ATCTCGGTCTCGACTTGGTGGACTTTCGACGTTCCCTGCTGGAGATGGCAGGGTGTGGTTGAGGTGGCTGCCACCTTCGGT
241 D N H L G G D D W D Q R V V D W L V D F K G T S G I D L T
721 GACAACCACTCCGGCGCACGACTGGGACCCAGCGGCTCGTGGCTGGCTGGAGAAGTCAAGGGACCCAGGGCATCGATCGACC
271 K D K M A M Q R L R E A A E K A K I E L S S S Q S T S I N L
811 AAGGACAAAGATGGCAGTCAGCGGCTGCGGGAAAGCCGAGAGGCAAAGATCGAGCTGAGTTCGAGTCAGTCCACCTCGATCAACCTG
301 P Y I T V D A D K N P L F L D E Q L T R A E F Q R I T Q D L
901 CCCTACATCACCGCTGACGGCAAGAACCCGTTCTTAGACGAGCCTGACCCGGCGGAGTCCACGGATCACTCAGGACCTG
331 L D R T R K P F Q S V I A D T G I S V S E I D H V V L V G G
991 CTGGACCCCACTCGCAAGCGCTTCAGTCGGTGATCGCTGACACCGCATTTCGGTGTGGAGATCGATCACTGGCTCGTGGGGT
361 S T R M P A V T D L V K E L T G G K E P N K G V N P D E V V
1081 TCCGACCCGGATGCCCGGGTGACCGAATCTGGTCAGGAACTCACCGCCGCAAGAACCCCAACAGGGCTCAACCCCGATGAGGTGTC
391 A V G A A L Q A G V L K G E V K D V L L L D V T P L S L G I
1171 GCGGTGGGAGCGGCTCTGAGGGCGCTCCCAAGGGCAGGTGAAGACGTTCTGCTGCTGATGTTACCCCGTGAACCTGGTATC
421 E T K G V M T R L I E R N T I P T K R S E T F T T A D D
1261 GAGACCAAGGGGGGGTGTGACCGAGGCTCATCGACCGAACACCCACGATCCACCCCAAGGGCTGGAGACTTACCCACCGCCGAGAC
451 N Q P S V Q I Q V Y Q G E R E I A A H N K L L G S F E L T G
1351 ACCAAACCGTCGGTGCAGATCCAGGTCTATCAGGGGGAGGGTGAAGATGCGCGCACACAACAACTGCTCGGGTCCCTCGAGCTGACCGC
481 I P P A P R G I P Q I E V T F D I D A N G I V H V T A K D K
1441 ATCCCGCCGCCGCCGGGGGATTCCGAGATCGACGGTCACTTCGACATGACGCCAACGGCATGTCACGTCAACCCCAAGGACAAG
511 G T K E N T I R I Q E G S G L S K E D I D R M I K D A E A
1531 GGCACCGCAAGGAGAACCGATCCGAATCCAGGAAGGGCTGGCCCTGTCACAGGAAGAACATTGACCGCATGATCAAGGACGCCAGCG
541 H A E D R K R E E A D V R N Q A E T L V Y Q T E K F V K
1621 CACCCCGAGGAGGATCGCAAGCGCTCGCAGGGCCATGTTCTTAATCAAGCGAGACATTGGCTACACAGCGAGAAGTTCGCTAAA
571 E Q R E A E G G S K V P E D T L N K V D A A V A E A K A A L
1711 CAAACAGCGTGAAGGGCGAGGGTGTGAAGGTACCTGAGACACGGCTGAACAGGGTGTGGCGCTGGCGAGCGAACGGGAGCATT
601 G G S D I A S I K S A M E K L G Q E S Q A L G Q A I Y E A A
1801 GGCGGATCGGATATTTCGGCCATCAAGTCGGCGATGGAGAAGCTGGCCAGGAGTCGAGGCTCTGGGCAAGCGATCTACGAAGCGACT
631 Q A A S Q A T G A A H P G G E P G G A H P G S A D D V V D A
1891 CAGGCTCGCTCACAGGCCACTGGCGTCCGCCACCCGGCGGAGCCGGGGCTGCCCACCCGGCTCGGCTGATGACGTTGGACCGG
661 E V V D D G R E A K *
1981 GAGGTGGTCGACGACGGCGGGAGGCCAAGTGA

```

Figure 1. Nucleotide and the aminoacid sequences of the $\text{A}\beta_{42}$ -Hsp70 protein. The $\text{A}\beta_{42}$ sequence (underlined), the linker aminoacid sequence (bold) and the Hsp70 protein sequence (remaining).

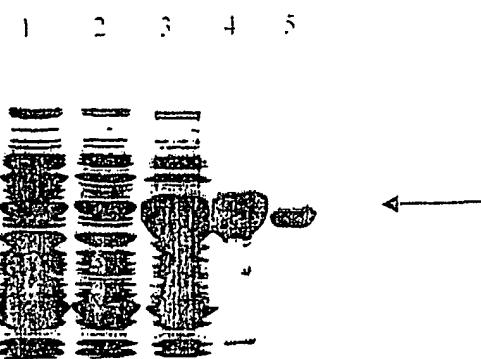


Figure 2. Production of recombinant protein. *E. coli* cell lysates and purified protein were analysed by SDS-PAGE and protein were visualised by Coomassie staining.

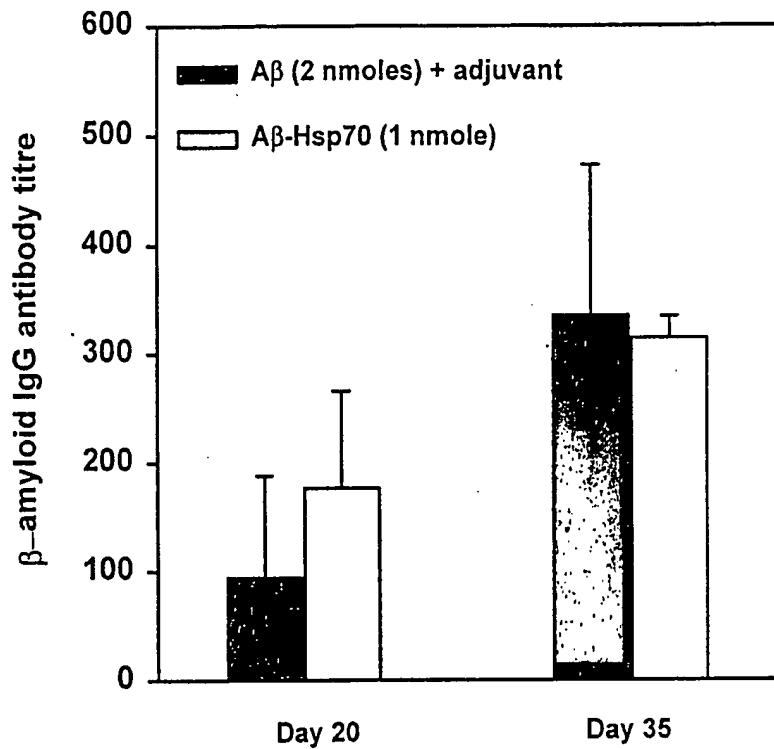


Figure 3. Anti-A β IgG antibody titre at day 20 and day 35 in mice injected on day 1, day 15 and day 30 with 2 nmoles of β -amyloid₁₋₄₂ (10 μ g) or 1 n mole of A β -Hsp70 fusion protein (100 μ g).

SEQUENCE LISTING

<110> CHIESI FARMACEUTICI S.P.A.

<120> FUSION PROTEINS AS IMMUNIZATION TREATMENTS OF
ALZHEIMER'S DISEASE

<130> CHIESI

<140>

<141>

<160> 6

<170> PatentIn Ver. 2.1

<210> 1.

<211> 2013

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: recombinant
Abeta42-Hsp70

<400> 1

atggatgcag aattccgaca tgactcagga tatgaagttc atcatcaaaa attgggtttc 60
tttgccaaag atgtgggttc aaacaaagggt gcaatcattg gactcatggt gggccgggtt 120
gtcatagccg gatccatggc tcgtgcggtc gggatcgacc tcgggaccac caactccgtc 180
gtctcgggttc tggaagggtgg cgacccgggtc gtcgtcgcca actccgaggg ctccaggacc 240
accccgtaa ttgtcgcgtt cgcccgcaac ggtgaggtgc tggtcggcca gccccccaag 300
aaccaggcag tgaccaacgt cgatcgacc gtgcgctcgg tcaagcgaca catgggcagc 360
gactggtcca tagagattga cggcaagaaa tacaccgcgc cggagatcag cgcccgatt 420
ctgtatgaaagc tgaagcgcga cgccgaggcc tacctcggtg aggacattac cgacgcgggtt 480
atcacaacgc cccctactt caatgacgc cagcgtcagg ccaccaagga cgccggccag 540
atcgccggcc tcaacgtgct gcgatcgac aacgagccgaa cccgcggccgc gctggcctac 600
ggcctcgaca agggcgagaa ggagcagcga atcctggctc tcgacttggg tggggcact 660
ttcgacgttt ccctgctgga gatcggcggag ggtgtgggtt aggtccgtgc cacttcgggt 720
gacaaccacc tcggccggcga cgactgggac cagcgggtcgc tcgattggct ggtggacaag 780
ttcaagggca ccagccgcatt cgatctgacc aaggacaaga tggcgatgca gcccgcgg 840
gaagccccc agaaggcaaa gatcgagctg agttcgagtc agtccacctc gatcaacctg 900
ccctacatca ccgtcgacgc cgacaagaac ccgttggct tagacgagca gctgaccgc 960
gcggagttcc aacggatcac tcaggacctg ctggacgcga ctgcgaagcc gttccagtcg 1020
gtgatcgctg acacccgcatt ttcgggtgcg gagatcgatc acgttgcgtc cgtgggtgg 1080
tcgaccggca tgccccgggt gaccgatctg gtcaaggaaac tcaccggcgg caaggaaccc 1140
aacaaggcgc tcaaccccgta tgagggtgtc gcggtggag ccgcgtctgca ggccggcgtc 1200
ctcaagggcg aggtgaaaga cgttctgctg cttgtatgttta ccccgctgag cctgggtatc 1260
gagaccaagg gcgggggtgat gaccaggcgc atcgagcgcac accaccaccaag 1320

cggtcggaga ctttacccac cgccgacgac aaccaaccgt cggtgagat ccaggtctat 1380
cagggggagc gtgagatcgc cgccgacaaac aagttgctcg ggtccttcga gctgaccggc 1440
atccccggcg cgccgcgggg gattccgcag atcgaggtca ctttcgacat cgacgccaac 1500
ggcattgtgc acgtcaccgc caaggacaag ggcacccggca aggagaacac gatccgaatc 1560
caggaaggct cgggcctgtc caaggaagac attgaccgca tgatcaagga cgccgaagcg 1620
cacggccgagg aggatcgcaa gctgcgcgag gaggccgatg ttctgtatca agccgagaca 1680
ttggtctacc agacggagaa gttcgtaaaa gaacagcggt aggccgaggg tgggtcgaaag 1740
gtacacctgaa acacgctgaa caagggttgc gccgcgggtgg cggaagcgaa ggccggactt 1800
ggcggatcgg atatttcggc catcaagtcg gctgatggaga agctggggcca ggagtcgcag 1860
gctctggggc aagcgatcta cgaagcgact caggctgcgt cacaggccac tggcgctgcc 1920
caccggcg gcgagccggg cgggtcccccac cccggctcggt ctgtatgcgt tggggacgcg 1980
gagggtgtcg acgacggcccg ggaggccaaag tga 2013

<210> 2

<211> 670

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: recombinant
Abeta42-Hsp70

<400> 2

Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln
1 5 10 15

Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile
 20 25 30

Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Gly Ser Met Ala Arg
35 40 45

Ala Val Gly Ile Asp Leu Gly Thr Thr Asn Ser Val Val Ser Val Leu
50 55 60

Glu	Gly	Gly	Asp	Pro	Val	Val	Val	Ala	Asn	Ser	Glu	Gly	Ser	Arg	Thr
65				70						75					80

Thr Pro Ser Ile Val Ala Phe Ala Arg Asn Gly Glu Val Val Leu Val Gly
85 90 95

Gln Pro Ala Lys Asn Gln Ala Val Thr Asn Val Asp Arg Thr Val Arg
100 105 110

Ser Val Lys Arg His Met Gly Ser Asp Trp Ser Ile Glu Ile Asp Gly
115 120 125

Lys Lys Tyr Thr Ala Pro Glu Ile Ser Ala Arg Ile Leu Met Lys Leu
130 135 140

Lys Arg Asp Ala Glu Ala Tyr Leu Gly Glu Asp Ile Thr Asp Ala Val
145 150 155 160

Ile Thr Thr Pro Ala Tyr Phe Asn Asp Ala Gln Arg Gln Ala Thr Lys
165 170 175

Asp Ala Gly Gln Ile Ala Gly Leu Asn Val Leu Arg Ile Val Asn Glu
180 185 190

Pro Thr Ala Ala Ala Leu Ala Tyr Gly Leu Asp Lys Gly Glu Lys Glu
195 200 205

Gln Arg Ile Leu Val Phe Asp Leu Gly Gly Thr Phe Asp Val Ser
210 215 220

Leu Leu Glu Ile Gly Glu Gly Val Val Glu Val Arg Ala Thr Ser Gly
225 230 235 240

Asp Asn His Leu Gly Gly Asp Asp Trp Asp Gln Arg Val Val Asp Trp
245 250 255

Leu Val Asp Lys Phe Lys Gly Thr Ser Gly Ile Asp Leu Thr Lys Asp
260 265 270

Lys Met Ala Met Gln Arg Leu Arg Glu Ala Ala Glu Lys Ala Lys Ile
275 280 285

Glu Leu Ser Ser Ser Gln Ser Thr Ser Ile Asn Leu Pro Tyr Ile Thr
290 295 300

Val Asp Ala Asp Lys Asn Pro Leu Phe Leu Asp Glu Gln Leu Thr Arg
305 310 315 320

Ala Glu Phe Gln Arg Ile Thr Gln Asp Leu Leu Asp Arg Thr Arg Lys
325 330 335

Pro Phe Gln Ser Val Ile Ala Asp Thr Gly Ile Ser Val Ser Glu Ile
340 345 350

Asp His Val Val Leu Val Gly Gly Ser Thr Arg Met Pro Ala Val Thr
355 360 365

Asp Leu Val Lys Glu Leu Thr Gly Gly Lys Glu Pro Asn Lys Gly Val
370 375 380

Asn Pro Asp Glu Val Val Ala Val Gly Ala Ala Leu Gln Ala Gly Val
385 390 395 400

Leu Lys Gly Glu Val Lys Asp Val Leu Leu Leu Asp Val Thr Pro Leu
405 410 415

Ser Leu Gly Ile Glu Thr Lys Gly Gly Val Met Thr Arg Leu Ile Glu
420 425 430

Arg Asn Thr Thr Ile Pro Thr Lys Arg Ser Glu Thr Phe Thr Thr Ala
435 440 445

Asp Asp Asn Gln Pro Ser Val Gln Ile Gln Val Tyr Gln Gly Glu Arg
450 455 460

Glu Ile Ala Ala His Asn Lys Leu Leu Gly Ser Phe Glu Leu Thr Gly
465 470 475 480

Ile Pro Pro Ala Pro Arg Gly Ile Pro Gln Ile Glu Val Thr Phe Asp
485 490 495

Ile Asp Ala Asn Gly Ile Val His Val Thr Ala Lys Asp Lys Gly Thr
500 505 510

Gly Lys Glu Asn Thr Ile Arg Ile Gln Glu Gly Ser Gly Leu Ser Lys
515 520 525

Glu Asp Ile Asp Arg Met Ile Lys Asp Ala Glu Ala His Ala Glu Glu
530 535 540

Asp Arg Lys Arg Arg Glu Glu Ala Asp Val Arg Asn Gln Ala Glu Thr
545 550 555 560

Leu Val Tyr Gln Thr Glu Lys Phe Val Lys Glu Gln Arg Glu Ala Glu
565 570 575

Gly Gly Ser Lys Val Pro Glu Asp Thr Leu Asn Lys Val Asp Ala Ala
580 585 590

Val Ala Glu Ala Lys Ala Ala Leu Gly Gly Ser Asp Ile Ser Ala Ile
595 600 605

Lys Ser Ala Met Glu Lys Leu Gly Gln Glu Ser Gln Ala Leu Gly Gln
610 615 620

Ala Ile Tyr Glu Ala Ala Gln Ala Ala Ser Gln Ala Thr Gly Ala Ala
625 630 635 640

His Pro Gly Gly Glu Pro Gly Gly Ala His Pro Gly Ser Ala Asp Asp
645 650 655

Val Val Asp Ala Glu Val Val Asp Asp Gly Arg Glu Ala Lys
660 665 670

<210> 3

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial
Sequence: oligonucleotidic primer

<400> 3

gggatccatg gtcgtgcgg tc

22

<210> 4

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial
Sequence: oligonucleotidic primer

<400> 4

ccgctcgagt cacttggcct ccccg

24

<210> 5

<211> 155

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial
Sequence: oligonucleotidic primer

<400> 5

cccaagcttc catatggatg cagaattccg acatgactca ggatatgaag ttcatcatca 60
aaaattggtg ttctttcag aagatgtggg ttcaaacaaa ggtgcaatca ttggactcat 120
ggtgtggcggt gttgtcatag cgggatccat ggctc 155

<210> 6
<211> 155
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial
Sequence:oligonucleotidic primer

<400> 6
gagccatgga tcccgtatg acaacaccgc ccaccatgag tccaatgatt gcaccttgt 60
ttgaacccac atcttctgca aagaacacca atttttgatg atgaacttca tatacctgagt 120
catgtcgaa ttctgcatcc atatgaaagc ttgggg 155

INTERNATIONAL SEARCH REPORT

National Application No

PCT/EP 01/12242

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 A61K39/385 A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P, X	WO 01 52890 A (UNIV CONNECTICUT HEALTH CT) 26 July 2001 (2001-07-26) the whole document ---	1-7
P, X	WO 01 52877 A (UNIV CONNECTICUT HEALTH CT) 26 July 2001 (2001-07-26) the whole document ---	1-7
E	WO 02 11669 A (ANTIGENICS LLC) 14 February 2002 (2002-02-14) the whole document ---	1-7
A	WO 94 29459 A (WHITEHEAD BIOMEDICAL INST) 22 December 1994 (1994-12-22) ---	
A	WO 95 31994 A (FRIDKIN MATITYAHU ;KONEN WAISMAN STEPHANIE (IL); COHEN IRUN R (IL)) 30 November 1995 (1995-11-30) ---	
		-/-

 Further documents are listed in the continuation of box C Patent family members are listed in annex

* Special categories of cited documents

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the International search	Date of mailing of the International search report
28 March 2002	08/04/2002
Name and mailing address of the ISA European Patent Office P B 5818 Patentlaan 2 NL 2280 HV Rijswijk Tel (+31-70) 340 2040 Fax 31 651 epo nl. Fax (+31-70) 340 2016	Authorized officer Cervigni, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/12242

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 99 42472 A (IGEN INT INC) 26 August 1999 (1999-08-26) -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No

PCT/EP 01/12242

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 0152890	A 26-07-2001	AU WO	2796001 A 0152890 A1	31-07-2001 26-07-2001
WO 0152877	A 26-07-2001	AU WO	2959701 A 0152877 A1	31-07-2001 26-07-2001
WO 0211669	A 14-02-2002	WO	0211669 A2	14-02-2002
WO 9429459	A 22-12-1994	AT DE EP JP WO US US	212378 T 69429723 D1 0700445 A1 8510756 T 9429459 A1 6338952 B1 6335183 B1	15-02-2002 14-03-2002 13-03-1996 12-11-1996 22-12-1994 15-01-2002 01-01-2002
WO 9531994	A 30-11-1995	AU AU CA EP JP WO US	684369 B2 2602795 A 2191202 A1 0760671 A1 10504524 T 9531994 A1 5869058 A	11-12-1997 18-12-1995 30-11-1995 12-03-1997 06-05-1998 30-11-1995 09-02-1999
WO 9942472	A 26-08-1999	US AU WO	6319503 B1 2470499 A 9942472 A1	20-11-2001 06-09-1999 26-08-1999